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Glucan-Induced Enhancement of Host Resistance to Selected Infectious Diseases

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We conducted studies with mice, rats, and monkeys which demonstrated the ability of glucan to induce either nonspecific or specific enhancement of host resistance to infectious diseases. Intravenous pretreatment of mice with glucan significantly enhanced the survival of mice challenged with either Venezuelan equine encephalomyelitis (VEE) virus or Rift Valley fever virus. Pretreatment was beneficial when initiated 3 days before challenge with VEE virus and 7 days before challenge with Rift Valley fever virus. Treatment of mice after VEE challenge did not increase their survival compared with controls. Glucan pretreatment of rats provided increased resistance to both intraperitoneal and low-dose aerosol challenges with virulent *Francisella tularensis* when the glucan was given intravenously, but not when it was administered intranasally. In contrast, intranasal glucan pretreatment enhanced the survival of mice when they were challenged by aerosol with *Pseudomonas pseudomallei*, whereas intravenous glucan pretreatment did not increase survival. Mice given glucan combined with a marginally immunogenic dose of VEE vaccine were more resistant to homologous virus challenge than were mice given either Freund complete adjuvant plus vaccine or vaccine alone. Similarly, both primary and secondary VEE antibody titers in cynomolgus monkeys given glucan with VEE vaccine were significantly greater than titers in vaccine controls.

The importance of the reticuloendothelial system and its primary component, macrophages, in mediating host resistance to infectious diseases was recognized some time ago. To paraphrase Mims (25), macrophages are literally in a position to control the susceptibility of animals to infectious diseases, since they monitor the main body compartments and may control the entry of infectious microbes to target organs. Numerous investigators have demonstrated the nonspecific (i.e., not immune-mediated) ability of macrophages to influence host susceptibility or lack thereof to a number of infectious processes (1, 14, 15, 18, 23, 27, 33; L. J. Old, D. A. Clarke, E. Stockert, C. Porter, and S. W. Orenski, Fed. Proc. **20**:265, 1961). Furthermore, only recently has the crucial role of macrophages in antigenic recognition and processing during the induction and amplification of specific immune responses been recognized (14). Apparently, macrophages not only are capable of modulating the response to an immunogen, but also can control what specific molecular sequence is recognized as an antigenic determinant (31).

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With the advent of numerous pharmacological agents which selectively enhance reticuloendothelial system function, the study of macrophage-mediated host resistance is both a fruitful and meaningful scientific endeavor. We used one such macrophage stimulant, yeast glucan, in the studies reported here.

Yeast glucan is extracted as a particulate compound from the inner cell walls of bakers' yeast (*Saccharomyces cerevisiae*) and has been shown to be a water-insoluble, β-1,3-glucopyranose polysaccharide with a helical ultrastructural configuration (6, 30). Glucan potentiates both cellular and humoral immunity to a diverse group of microbial and tumor antigens and nonspecifically enhances host resistance to neoplastic, bacterial, and fungal diseases (6–10; N. R. Di Luzio, W. Browder, R. McNamee, and J. A. Cook, Fed. Proc. **36**:1242, 1977). Its effects can be categorized as macrophage-mediated immunostimulation. The purposes of this study were (i) to examine the immunomodulating activities of glucan on the nonspecific enhancement of host resistance to infectious diseases by using primary pathogens and (ii) to assess the adjuvant activity of glucan when it is administered with inactivated viral antigens.

MATERIALS AND METHODS

Animals. Outbred male mice (either CD-1 [Charles River Breeding Laboratories, Wilmington, Mass.] or strain ICR [Edgewood Arsenal, Edgewood, Md.]) weighing 20 to 25 g were housed in steel, closed-bottom cages with hardwood chip bedding. Male, inbred Fischer-344 rats (Harlan Laboratories, Indianapolis, Ind.) weighing 200 to 225 g were housed in polycarbonate cages with the same bedding. All rodents were fed a commercial diet (Purina Rodent Chow; Ralston-Purina Co., St. Louis, Mo.) and given water ad libitum. Monkeys were healthy, mature, laboratory-conditioned cynomolgus monkeys (*Macaca fascicularis*; both sexes) which were seronegative for Venezuelan equine encephalomyelitis (VEE) virus-neutralizing antibody. Monkeys were housed individually in suspended metal cages and fed a commercial monkey diet (Purina Monkey Chow) twice daily and provided water ad libitum. The monkey diet was supplemented three times a week with fresh fruit. All animals were kept in rooms at a constant temperature (22°C) with a 12-h light-dark cycle.

Glucan. Endotoxin-free glucan was prepared by a modification of the procedure of Hassid et al. (16) and was supplied as a sterile suspension either in a 0.9% NaCl solution or in water. The concentration was adjusted as needed by using sterile pyrogen-free saline or water.

VEE antigen. The Formalin-inactivated VEE vaccine used in these studies has been described previously (5). All vaccine dilutions were made with sterile pyrogen-free saline.

Challenge viruses. The Trinidad strain of VEE virus was isolated in guinea pigs from a donkey in Trinidad. In addition to the guinea pig passage, there had been 13 serial passages in embryonated chicken eggs, 1 passage in duck embryo cells, and 1 final passage in chicken fibroblasts. This virus strain produces a fatal paralytic disease in mice when inoculated subcutaneously or intraperitoneally (i.p.). Inocula were diluted before challenge with phosphate-buffered saline containing 1% normal, heat-inactivated rabbit serum. The virus challenge dose was titrated by inoculating serial 10-fold dilutions into nonvaccinated mice and calculating the 50% lethal dose, as previously described (29).

The Zagazig 501 strain of Rift Valley fever (RVF) virus was isolated in 1977 by James Meegan, Cairo, Egypt, from a fatal human case of RVF with hemorrhagic manifestations. Virus from human serum was passaged twice in diploid fetal rhesus monkey lung (FRhL-103) cells. Inocula were diluted in Hanks balanced salt solution containing 2% normal, heat-inactivated fetal bovine serum.

Challenge bacteria. Virulent *Francisella tularensis* strain SCHU S4, which was used for aerosol and i.p. challenges, was prepared and stored at −60°C (12). The aerosol challenge dose was calculated on the basis of the concentration of the organism per liter of aerosol, the duration of exposure, and the tidal volume of the animal (20).

Pseudomonas pseudomallei ATCC 23343 was used. Its virulence for mice was enhanced by 20 serial intracranial passages, followed by 5 i.p. passages in mice (28). After passage, cultures were incubated in Trypticase soy broth (pH 6.8) at 37°C for 48 h and stored in 5-ml portions at −60°C. When thawed, the cultures contained 2×10^9 colony-forming units per ml.

Serum neutralizing antibody determinations. VEE serum neutralizing antibody titrations were performed in triplicate (11) by using Vero cells grown in six-well plastic trays. Starting with an initial dilution of 1:4, serial twofold dilutions were made in Hanks balanced salt solution. The greatest serum dilution giving 80% plaque reduction was selected as the endpoint. Endpoints below 1:4 were assigned a value of 1:2 to calculate the group geometric mean titer.

Statistics. Chi-square analysis with Yate's correction was used to compare the mortality data of test groups with their controls. One-way analysis of variance was used for intergroup comparisons of antibody data.

RESULTS

Nonspecific potentiation of resistance. (i) VEE virus challenge. ICR mice were placed into four experimental groups containing 60 mice each (Fig. 1). Mice in groups I through III were given 4 mg of glucan per 100 g of body weight in 0.1 ml intravenously (i.v.) at the following times: group I, days −3 and 0; group II, days 0 and 3; group III, days 1 and 4. The mice in group IV were given saline on days 0 and 1. On day 0, 20 animals in each group were challenged i.p. with 8,000, 800, or 80 mouse i.p. 50% lethal doses of VEE virus. Mice were observed daily for mortality, and percent survival for each group was calculated on day 21 postchallenge.

Glucan treatment given i.v. either on days 0 and 3 or on days 1 and 4 postinfection did not provide enhanced protection from virus challenge compared with the saline controls. In contrast, glucan pretreatment on days −3 and 0 did provide significant nonspecific protection from challenge with either 800 or 80 mouse i.p. 50% lethal doses of virulent VEE virus (Fig. 2). These glucan-treated groups had 27 and 35% survival

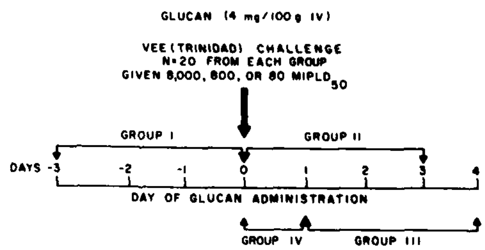

FIG. 1. *Experimental design to assess the ability of glucan to potentiate nonspecifically the resistance of mice to VEE virus challenge. MIPLD$_{50}$, Mouse i.p. 50% lethal doses.*

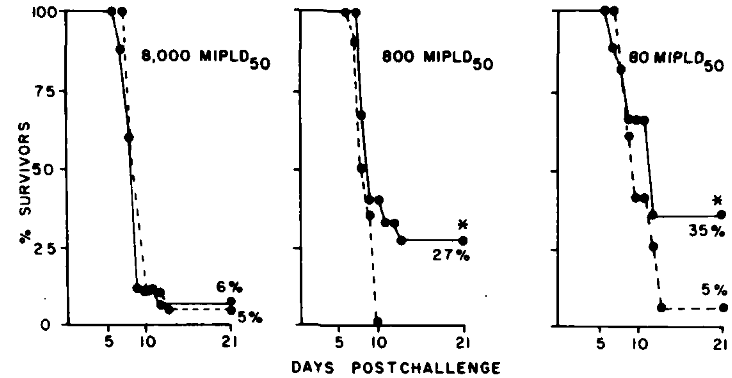

FIG. 2. *Resistance of glucan-treated mice (——, 4 mg/100 g) to challenge with various doses of VEE virus. MIPLD$_{50}$, Mouse i.p. 50% lethal doses. - - -, Saline; *, $P < 0.05$.*

rates, respectively, compared with 0 and 5% survivors in their corresponding controls.

(ii) RVF virus challenge. CD-1 mice were placed into treatment groups of at least 40 mice each. One group was given 5.2 mg (0.1 ml) of glucan per 100 g of body weight i.v. on days −7, −4, and −1, and another group was given only saline. Mice were challenged subcutaneously on day 0 with either 100 or 250 plaque-forming units of RVF virus. All mice were observed daily, and survival was determined on day 21.

Because there was no difference in survival between the two virus challenge doses, results were pooled (Table 1). There was a significant potentiation of resistance to challenge with virulent RVF virus in glucan-treated mice compared with controls. Survival in treated mice was 84%, compared with 50% in controls.

(iii) *F. tularensis* challenge. Inbred Fischer-344 rats were divided into experimental groups (Table 2). They were treated with glucan (1 mg/100 g of body weight) either i.v. or intranasally (i.n.) on days −5, −3, and −1. The group treated i.n. was anesthetized with halothane (Fluothane; Ayerst Laboratories, New York, N.Y.) before inoculation of the calculated glucan dose in 0.1 ml. Rats were challenged with virulent *F. tularensis* by either i.p. inoculation of 10^5 cells or exposure to 10^5 or 10^3 cells in a small-particle aerosol delivered by a modified Henderson tube (20). Mortality rates were calculated on day 21 postinfection.

Mortality of untreated rats was 100% after i.p. inoculation and 62% after either high- or low-dose aerosol exposure. In contrast, i.v. pretreatment with glucan significantly increased survival of rats challenged both by the i.p. route and by a low-dose aerosol exposure (Table 2). However, glucan given i.n. did not increase survival in animals challenged i.p.; i.n. glucan pretreatment did increase survival in the low-dose aerosol challenge group, but this enhanced protection was not statistically significant.

(iv) *P. pseudomallei* challenge. ICR mice

TABLE 1. *Effects of glucan pretreatment on survival of mice challenged subcutaneously with RVF virus*[a]

Treatment[b]	No. of survivors/ total no.	% Survival on day 21	P
Glucan	36/43	84	<0.005
Saline	20/40	50	

[a] Mice were challenged on day 0 with 100 or 250 plaque-forming units of RVF virus.

[b] Glucan (5.2 mg/100 g of body weight in 0.1 ml) was given i.v. on days −7, −4, and −1, or 0.1 ml of saline was given i.v. on days −2, −1, and 0.

TABLE 2. *Effect of glucan pretreatment on survival of rats infected with F. tularensis*

Treatment	Route[a]	Infection	Survival: No./total no.	Survival: %	P
Glucan	i.v.	None	24/24	100	
	i.n.	None	16/16	100	
None		i.p.	0/16	0	
Glucan	i.v.	i.p.	11/16	68	<0.0001
	i.n.	i.p.	0/8	0	
None		Aerosol (10^5 cells)	3/8	38	
Glucan	i.v.	Aerosol (10^5 cells)	3/16	19	
	i.n.	Aerosol (10^5 cells)	3/8	38	
None		Aerosol (10^3 cells)	6/16	38	
Glucan	i.v.	Aerosol (10^3 cells)	16/16	100	<0.001
	i.n.	Aerosol (10^3 cells)	10/16	63	

[a] Glucan (1 mg/100 g of body weight) was given i.v. via the tail vein or i.n. (total volume, 0.1 ml) on days −5, −3, and −1.

were assigned to experimental groups (Table 3). They were treated with 2.0 mg of glucan per 100 g of body weight in 0.1 ml either i.v. or i.n. on days −7, −4, and −1. Control mice received only 0.1 ml of sterile water on the same days and by the same routes. Mice treated i.n. were anesthetized with halothane before instillation of glucan or water. Mice were each challenged in the Henderson tube on day 0 with 30 aerosolized mouse-adapted *P. pseudomallei* cells. Mortality rates were calculated on day 21 postinfection.

The effects of a glucan pretreatment on the survival of mice challenged with *P. pseudomallei* are shown in Table 3. Glucan given i.n. significantly increased survival of infected mice compared with controls treated with water i.n. Despite the 40% survival rate in the i.n. water-treated and infected controls, the 85% survival rate in the i.n. glucan-treated group was still highly significant ($P < 0.001$). The reason for the increased survival of the i.n. water-treated animals cannot be explained by the present data. Glucan given i.v. did not enhance the resistance of aerosol-challenged mice significantly.

Specific enhancement of resistance. (i) VEE virus challenge. This experiment was done to evaluate the adjuvant activity of glucan in mice when the glucan was combined with a marginally immunogenic dose of VEE virus vaccine. Male CD-1 mice were placed into four groups of 16 mice each and inoculated subcutaneously with 0.3 ml (1:4 dilution) of VEE vaccine combined with either 2.0 mg (0.1 ml) of glucan per 100 g of body weight, Freund complete adjuvant (1:1 ratio with vaccine), or an equivalent volume of saline (Table 4). The control group received 0.4 ml of saline.

All mice were challenged i.p. on day 21 post-

TABLE 3. *Effect of glucan pretreatment on survival of mice infected with mouse-adapted P. pseudomallei by small-particle aerosol*

Treatment	Route[a]	Infection	Survival: No./total no.	Survival: %	P[b]
None		+	7/40	18	
Glucan	i.v.	−	40/40	100	
		+	11/40	28	
Glucan	i.n.	−	40/40	100	
		+	34/40	85	<0.001
Water	i.v.	−	20/20	100	
		+	2/20	10	
Water	i.n.	−	20/20	100	
		+	8/20	40	

[a] All treatments were given on days −7, −4, and −1. Glucan (2.0 mg/100 g of body weight) was given in a total volume of 0.1 ml.

[b] Compared with i.n. water-treated and infected group.

TABLE 4. *Adjuvant effects of glucan combined with VEE vaccine given subcutaneously to mice which were then challenged at 21 days with virulent VEE virus*

Treatment[a]	% Survival ($n = 16$)	P
VEE + glucan	81	<0.05
VEE + saline	38	
VEE + Freund complete adjuvant (1:1)	6[b]	
Saline control	6	

[a] VEE, 0.3 ml of vaccine (1:4 dilution); glucan, 2.0 mg/100 g of body weight.

[b] The decreased survival rate of the group given Freund complete adjuvant was not significantly different than the survival rate of vaccine controls ($P < 0.01$).

vaccination with 425 mouse i.p. 50% lethal doses of virulent VEE virus and were observed for 21 days for mortality. Mice given glucan plus vaccine were significantly more resistant to homologous virus challenge (Table 4) than were mice given either vaccine alone or vaccine emulsified in Freund complete adjuvant. The glucan-treated group had 81% survivors, compared with 38% for the vaccine controls.

(ii) VEE serum neutralizing antibody production. This study assessed the ability of glucan, when given in combination with VEE vaccine, to enhance both primary and secondary antibody responses to VEE virus in cynomolgus monkeys. Three groups of four monkeys each were inoculated intramuscularly on day 0 and boosted on day 28 with 0.5 ml of VEE vaccine alone or VEE vaccine combined with either 0.5 or 1.0 mg of glucan per 100 g of body weight. All monkeys were bled at weekly intervals for serum neutralizing antibody determinations.

The geometric mean serum neutralizing antibody titers for all groups of monkeys over an 87-day period are shown in Fig. 3. The antibody titers in the three groups during the first 21 days post-inoculation were not significantly different. However, on day 28 the titers in monkeys given 1.0 mg of glucan per 100 g of body weight combined with vaccine were significantly ($P < 0.05$) greater than the titers in the vaccine controls.

After boosting on day 28, the antibody titers in monkeys given 1.0 mg of glucan per 100 g of body weight plus vaccine remained significantly elevated compared with the vaccine controls for at least 52 days. In contrast, the antibody responses of monkeys after two doses of vaccine combined with 0.5 mg of glucan per 100 g of body weight were significantly elevated only on days 42 through 56 compared with the titers in the vaccine controls. On day 87, when the experiment was terminated, the antibody titers of monkeys given two injections of vaccine combined with either 1.0 or 0.5 mg of glucan per 100

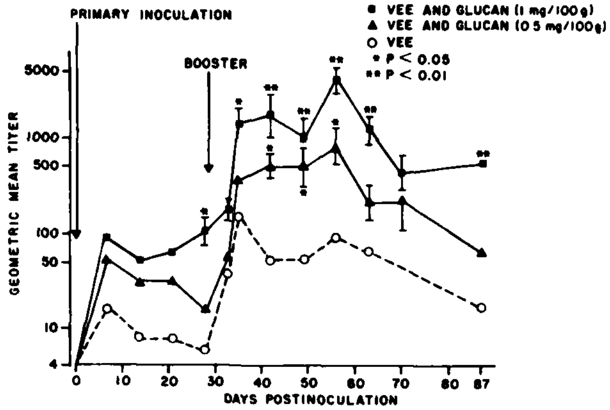

FIG. 3. *Primary and secondary antibody responses of cynomolgus monkeys immunized intramuscularly with VEE vaccine plus glucan.*

g of body weight were 22-fold (1:512) and 3-fold (1:64) higher, respectively, than the titers in the vaccine controls (1:23).

DISCUSSION

We utilized glucan to alter nonspecifically the course of several lethal experimental infections in mice and rats. In addition, we demonstrated the ability of glucan to enhance specifically the resistance of mice to homologous virus challenge and to potentiate both primary and secondary antibody responses in nonhuman primates. Since glucan has been shown by others to induce readily both hyperplasia and hyperfunction of the reticuloendothelial system (2, 3, 9, 36), it is our assumption that this enhanced nonspecific resistance and this specific resistance were due primarily to macrophage-mediated events.

The possible role of glucan-induced interferon as a mediator of the increased nonspecific resistance to both VEE and RVF virus challenges seen in our studies was not examined. Since glucan is now known to be an interferon inducer (Di Luzio, unpublished data), this explanation seems likely. In addition, pretreatment of mice with glucan before challenge with a lethal virus infection may create an environment for a more efficient induction of interferon by the virulent challenge virus. This priming or augmentation of interferon production has been noted by others both in vivo and in vitro, using various synthetic interferon inducers and viruses (13, 32).

The pathogeneses of both RVF virus and VEE virus infections have a significant macrophage component (19, 24, 25). The phagocytic cells of the reticuloendothelial system are uniquely situated to provide a functional blockade to virus infection of hepatocytes or other target organs (25, 26, 34). Indeed, our studies suggest that by enhancing macrophage function with glucan, the reticuloendothelial system is in an as-yet-undefined manner capable of effectively reducing the pathogenicity of VEE and RVF virus infections in mice. An examination of the biochemical and cellular events responsible for this glucan-induced enhanced resistance would be a worthwhile endeavor.

The ability of macrophages to alter the fate of intracellular microorganisms, especially bacteria, has been reported (15, 23), although the precise mechanisms are not entirely understood. Most studies demonstrating glucan-induced immunopotentiation have dealt only with infectious agents that are primarily opportunistic (e.g., *Staphylococcus* spp., *Candida*, sporotrichosis) and with noninfectious neoplastic diseases (7, 8, 10, 22). We have demonstrated modifications in the courses of selected infectious diseases by glucan treatment by using highly virulent bacteria and viruses.

Data from our studies with bacterial infections suggest that the efficacy of glucan treatment may vary depending upon its route of administration and the specific host-parasite interaction. An experimental tularemia infection of rats results in a systemic dissemination of virulent organisms within 24 h after i.p. inoculation and within 48 h after aerosol challenge (Jemski, unpublished data). Prior activation of the reticuloendothelial system by i.v. glucan pretreatment resulted in a marked potentiation of resistance to i.p. tularemia infection, whereas local deposition of glucan in the pulmonary tract did not increase significantly resistance to an overwhelming respiratory or systemic challenge with virulent organisms.

Conversely, respiratory melioidosis, which

usually does not become systemically disseminated until the terminal stages of the disease, did not respond to i.v. glucan pretreatment. However, local administration of glucan to the respiratory tract significantly enhanced the resistance of mice challenged by aerosol. *P. pseudomallei* is not an obligate intracellular pathogen and readily proliferates in the pulmonary alveoli and bronchioles. The extracellular growth of these organisms is apparently markedly suppressed by activated macrophages, perhaps by the increased production of extracellular enzymes (22) or bacteriostatic products (17).

Numerous compounds, including BCG (18, 33), Bru-pel (37), *Corynebacterium parvum* (21), muramyl dipeptide (4), and levamisol (21), which act predominately to enhance macrophage function, have also been shown to enhance the immunogenicity of microbial antigens. The ability of glucan to potentiate humoral antibody responses (35) and specific protective immunity when given as an adjuvant in the above-described studies is most easily explained by glucan enhancement (i.e., macrophage-mediated amplification) of immune induction.

We did not evaluate systemic morphological changes induced in our studies. Obviously, before the use of macrophage stimulants like glucan would be clinically acceptable, additional work (7) would be required to demonstrate that glucan is nonreactogenic and nontoxic and does not induce irreversible systemic morphological changes when given at effective dosage levels.

The true benefit of macrophage stimulants, such as glucan, may reside not only in their adjuvant activitiy, but also in their ability to maintain nonspecific resistance to infectious diseases while a host is temporarily immunosuppressed, as is the case with cancer chemotherapy and radiation injuries.

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